Supplement 1: Description of the different assays to detect minority variants of drug-resistant HIV-1

Point mutation assays

Line probe assay (LiPA) [17, 18]: This assay exploits differential hybridization of HIV-DNA fragments with specific capture oligonucleotides that are immobilized on a nitrocellulose membrane in a strip format. Viral target sequences containing the variants of interest are complementary DNA (cDNA) that has been reverse transcribed from RNA, and then amplified by PCR. The differential binding of these target sequences to the sequence-specific oligonucleotides is then detected with a probe that should recognize the target sequences.

Allele specific real-time polymerase chain reaction (AS-PCR) [19-27]: AS-PCR selectively amplifies either the wild type codon or a mutant sequence using specific oligonucleotide primers, detecting them with qPCR. This assay based on the Amplification Refractory Mutation System (ARMS) described by Newton in 1989 [28] was first used in the setting of HIV by Larder in 1991 to detect zidovudine resistant strains [29].

MultiCode-RTx [30, 31]: This assay also uses sequence specific primers but with an additional modified base-pair to incorporate a quencher in close proximity to a fluorescent molecule at the 5' end of the target specific primer. The number of qPCR cycles required to observe reduction of fluorescence below a specific threshold is proportional to the initial target concentration.

Oligonucleotide ligation assays (OLA) [32-37]: This method is based on selective hybridization of two oligonucleotides varying by a base at the nucleotide of interest, followed by a ligase reaction on a PCR derived patient sample. Allele discrimination results from the ability of DNA-ligase to join only perfectly matched probes. By marking each of the allele-specific primers with a unique hapten, the target allele is identified with hapten-specific-antibodies that are labeled with different enzyme reporters.

LigAmp [38, 39]: Similar to OLA, the LigAmp assay utilizes mutation-specific ligation of two selective oligonucleotides with a DNA-template. Each oligonucleotide contains a region specific to the target gene and a tail that permits primer-hybridization and subsequent amplification of the ligated product. The upstream oligonucleotide also contains a region that serves as binding site for a probe, detected in a subsequent qPCR reaction.

Rolling circle amplification (RCA) [40, 41]: This method exploits “padlock” probes with consecutive signal amplification by RCA. These circular allele specific probes hybridize with the target in a “head-to-tail” manner, and following incubation with DNA-ligase, they form closed circular molecules. A mismatch between the open circle probe and the target DNA prevents ligation and circularization. This circularized probe can be amplified and detected in a qPCR instrument.

Sequencing assays

Single genome sequencing (SGS) [42, 43]: This approach relies on sequences of PCR product obtained from the amplification of single DNA or cDNA molecules after template limiting-dilution. It is critical that the sample material contains a population size to permit representative sampling and that the primers do not confer selective amplification. If these conditions are met, this approach provides a "gold standard" for quantification of minority variants. Unfortunately this approach is slow, expensive and labor-intensive.

Clonal sequence analyses [12, 15, 44, 45]: This approach allows sequencing of several viral variants from a single plasma sample. After HIV-RNA isolation from patient plasma, cDNA is synthesized in a standard reverse transcription reaction. The region of interest is subsequently PCR amplified and ligated into a plasmid vector. Ligation products are introduced into competent cells, usually Escherichia coli by transformation. Transformed bacterial colonies provide the DNA for sequencing. Theoretical concerns include the risk of recombination during the initial amplification, although one study documenting recombination of DRMs in cell culture ruled out this artifact of amplification [46]. The assay conditions also must be designed to allow for proper sampling to represent the true diversity of the population being analyzed.

Heteroduplex tracking assay (HTA) [47, 48]: A radioactively or enzymatically labeled probe is annealed to a PCR product (derived from the total viral population) in order to generate specific probe-PCR product heteroduplexes,

which can be separated by gel electrophoresis. The specificity for detecting point mutations is introduced into the probe by changing nucleotides close to the sites of interest. Presence of mutations results in differential migration of the heteroduplex, allowing separation of distinct subpopulations. After isolation of the obtained band from the gel and suspension in PCR mix, separated viral variants can be re-amplified and sequenced.

Successive specific amplifications (SSA) [49]: This assay is based on the progressive amplification of minority variants using specially designed sets of specific primers, followed by clonal analyses of the selected subpopulations ("quasispecies diving"). This technique can identify minority species sensitively but without the ability of quantify.

Ultra-deep pyrosequencing (UDPS) [50-54]: UDPS allows parallel amplification and pyrosequencing of up to 100,000 or more individual DNA or cDNA molecules per single run. In a picoliter-scale sequencing reaction, DNA molecules are bound to beads under conditions favoring binding of single DNA molecules. Each bead is then loaded into single microwells and subjected to PCR amplification. All the obtained clonal sequences are aligned and analyzed to evaluate the prevalence of minority variants. This very sensitive method has been successfully used for the simultaneous detection of HIV-1 minority quasispecies in plasma samples. Although hundreds of thousands of sequences can be generated, specificity is limited by the fidelity of the PCR-amplification and

pyrosequencing, as well as the number of copies in the specimen being interrogated. Prevalence rates below 1% cannot be reliably detected [54, 55].

DNA microarrays [13, 56]: This technique is based on the covalent immobilization of tens of thousands of distinct single-stranded DNA-oligonucleotides (capture probes) on a solid substrate, like silicon chips. Viral target molecules that have been amplified and fluorescently labeled hybridize with the specific surface-bound complementary probe, and are detected with high-resolution scanning. Although this technology allows characterizing a large number of mutations in a single experiment, the genetic variability around the known target mutation of HIV may interfere with accuracy of target-probe hybridization, consequently reducing sensitivity of this assay. Although this technique can detect the simultaneous presence of codon mixtures, it is not quantitative, the covalent linkage between mutations cannot be discerned, and it overlooks insertions and deletions.

Parallel allele-specific sequencing (PASS) [57]: In this assay, a *pol*-gene fragment, containing all major DRMs, is amplified within a polyacrylamide gel matrix. Because one of the primers is covalently linked to the matrix, PCR-products accumulate around individual DNA-templates at the amplification site. Different viral variants are detected by differential incorporation of fluorescence-labeled nucleosides at the resistance-sites of interest.

Phenotypic assays

Enhanced-sensitivity trofile assay® [58, 61-64]: A population of full-length *envs* from patient plasma is first amplified and integrated in vectors, in order to create a library representing the patient's viral *env* population. In a second step, this *env* population is co-transfected together with an *env*-deleted luciferase-reporter HIV-plasmid to generate a mixture of pseudo-viruses carrying the patient-derived *env* proteins. These pseudo-viruses are subsequently added to target-cells expressing CD4 and either the CCR5 or the CXCR4 co-receptor. Infection of target-cells is assayed by the addition of luciferase-substrate and quantification of luminescence.

Phenotypic assays based on Saccharomyces cerevisiae (TyHRT) [39, 60]: This yeast-based assay allows screening of extensive libraries of HIV-reverse transcriptase containing clones for their activity and susceptibility against non nucleoside reverse transcriptase inhibitors (NNRTI). These hybrid elements are composed by *Saccharomyces cerevisiae* Ty1 retrotransposons in which reverse transcriptase is provided by HIV-1 (TyHRT). TyHRT-elements generate a very high frequency of reverse transcriptase mediated events, allowing characterization of reverse transcriptase activity over a 10,000-fold range. Since reverse transcriptase activity is inhibited by NNRTI in yeast [65], performing the assay in the presence of these inhibitors permits the determination of the drug resistance phenotype of each clone of the mixed viral population.